CesA protein is included in the terminal complex of Acetobacter

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CesA protein is included in the terminal complex of *Acetobacter*

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Running title: CesA in bacterial terminal complex

Abstract

Cellulose is a major biopolymer on the earth that is derived from cellulose synthase in the cell membrane of living organisms. Cellulose synthase is a hetero-subunit complex composed of several different protein subunits, and is visualized as a supermolecular complex called a “terminal complex” by electron microscopy. Such supermolecular organization of an enzyme complex is believed to be important for the fiber formation or crystallization of cellulose microfibrils in cellulose biosynthesis. In the case of the cellulose-producing bacterium *Acetobacter*, it is hypothesized that the enzyme complex includes at least six subunits given its genetic constitution. However, to date, only three of these molecules have been experimentally confirmed as the subunits included in the cellulose synthase complex: CesB, CesD, and ccp2. In this study, we used fluorescence immuno-microscopy to show that CesA protein, the catalytic subunit, is included in the terminal complex of *Acetobacter*. Furthermore we discuss the obtained microscopic data for improving our understanding of the molecular organization of the bacterial cellulose synthase complex.

Keywords: CesA; cellulose synthase complex; terminal complex; immunolabeling

Abbreviations: PFA, paraformaldehyde; EDTA, ethylenediamine tetra-acetic acid; SDS, sodium dodecyl sulfate; PAGE, poly-acrylamide gel electrophoresis; PVDF, poly-vinylidene difluoride; RT, room temperature; BSA, bovine serum albumin

INTRODUCTION

Cellulose is one of the major biopolymers on Earth. Despite its mass production on Earth by plants and other living organisms, destructive accumulation of cellulose on Earth has never been identified, although this has been observed for some synthetic plastics. This indicates that the cycle of synthesis and degradation shows a good balance for maintaining the cellulose content on Earth, which is actually a striking feature of cellulose, suggesting it as a promising material for sustainable human life.

All of the cellulose on Earth is produced by living organisms, and originates from the cellulose synthase complex (CSC) in living cells. CSC is a hetero-subunit complex in the cell membrane (Somerville 2006). Electron microscopy with the freeze-fracture technique has been used to visualize CSC as a terminal complex (TC), which is a characteristic array of particles found on the cell membrane at the terminal of cellulose microfibrils (Kimura et al. 1999, Kimura et al. 2001). Since the 1970s, TCs have been found in many of the cellulose-producing organisms (a tunicate, algae, plants, and a bacterium), although the arrangement of the TC particles showed a variety of patterns (Itoh et al. 2007): linear type (for a bacterium, tunicate, and algae producing a giant microfibril) and rosette-type (for higher plants and an alga of the order *Zygnematale*). Regardless of the specific pattern, such a regular array of a cellulose-synthesizing enzyme is considered to be important for cellulose microfibril formation by assembling many cellulose chains into a cellulose microfibril.

To date, several studies have identified the molecules included in the CSC based on biochemical and molecular/cell biological analyses. For *Acetobacter*

(recently renamed for some strains as *Gluconacetobacter*, *Komagataeibacter*, and so on), a popular model for studying cellulose biosynthesis, six subunits are proposed to be included in the CSC given the constitution of the genes related to cellulose synthesis (McNamara et al. 2015): GH-8 (also known as carboxymethyl cellulase (CMC)) (Standal et al. 1994), cellulose complementing factor (ccp) (Standal et al. 1994), CesA, CesB, CesC, and CesD (Saxena et al. 1994, Wong et al. 1990). Among these, CesA is the catalytic subunit harboring the glycosyltransferase domain of the GT-2 family in the cytosolic part (Morgan et al. 2013), and CesA and CesB are the minimally required subunits for cellulose-synthesizing activity (Omadjela et al. 2013, Saxena et al. 1994, Wong et al. 1990). CesD is considered to control the crystallization process of cellulose microfibrils (Hu et al. 2010, Saxena et al. 1994), and four chains are found inside the ring structure formed by the octamer of CesD protein (Hu et al. 2010). The functions of the other subunits have not yet been clarified despite their clear relevance to cellulose-synthesizing activity, as experimentally reported for GH-8 (Kawano et al. 2002, Kawano et al. 2008, Nakai et al. 2013), ccp (Sunagawa et al. 2013), CesC (Saxena et al. 1994), and CesD (Hu et al. 2010, Saxena et al. 1994, Sunagawa et al. 2013).

The SDS-freeze replica labeling (SDS-FRL) method (Fujimoto 1995), an immuno-labeling technique combined with the freeze-replica technique, is a direct method to localize a specific protein in the TCs, and its application has shown that CesB protein is found in the linear TC of *Acetobacter* (Kimura et al. 2001). In addition, fluorescence microscopy could also be used to successfully visualize the linear localization of GFP-fused CesD and ccp protein in the cells of *Acetobacter* (Sunagawa et al. 2013), which confirmed that these two proteins are also included

in the linear TC of *Acetobacter*. These microscopic studies showed that CesB, CesD, and ccp proteins form a part of the TC or CSC of *Acetobacter*. However, no report has provided concrete evidence that CesA protein is included in the linear TC of *Acetobacter*, although this is the widely accepted hypothesis given that CesA is the core subunit of the CSC. In the present study, CesA protein was successfully visualized as a linear array in the cell, and experimental evidence was obtained to show that CesA protein is included in the TC of *Acetobacter*.

MATERIALS AND METHODS

Chemicals

Peptone and yeast extract for the culture medium were purchased from Becton, Dickinson and Company Inc. (USA). Paraformaldehyde (PFA) for cell fixation was paraformaldehyde EM from TAAB Inc. (UK). Poly-L-lysine solution, lysozyme and BSA were purchased from Sigma-Aldrich Inc. The other chemicals used in this study were purchased from Wako Pure Chemicals Inc. (Japan) unless described.

Cell culture

Three different strains of *Acetobacter* were used in this study: ATCC53264, ATCC53524, and JCM9730. For convenience, the former name *Acetobacter* is used for these strains herein, although these are actually considered to be different species based on the current taxonomy (*Gluconacetobacter xylinus* for ATCC53264, *Komagataeibacter xylinus* for ATCC53524, and *Komagataeibacter sucrofermentans* for JCM9730). The two ATCC strains were provided by the American Type Culture Collection and the last strain was obtained from the Japan Collection Microorganisms at BRC-RIKEN, Japan. Each

strain was grown in Schramm-Hestrin medium (Schramm and Hestrin 1954) at 30°C in a static condition for 3 to 5 days, until a sufficient amount of cellulose was produced. The cells were detached from the cellulose pellicle by shaking the culture medium and pressing the pellicle with a spatula, and then filtered by 37- or 50-μm pore-sized nylon mesh. The filtrated cells were collected by centrifugation (2000×*g* for 10 min at RT).

Antibody evaluation by western blot analysis

Western blot analysis was performed to evaluate whether the primary antibody has cross-reactivity with the proteins in the strains ATCC53264 and JCM9730, as well as strain ATCC53524 for which cross-reactivity has already been shown (Hashimoto et al. 2011). The primary antibodies used in this study were the same as those used in our previous studies (Hashimoto et al. 2011, Imai et al. 2014, Sun et al. 2016). In brief, each antibody is a polyclonal antibody against the synthetic peptide corresponding to a part of CesA (carboxyl terminal), CesB (a loop in the CBD2 domain), CesC (the part between the last six-TPR repeat and the carboxyl terminal region), and the CesD subunit (the loop between the β3 and β4 strands). The antigen peptide sequence for each of the proteins was designed from the sequence of the strain BPR2001 (Nakai et al. 1998) or JCM9730 (GenBank: AB010645) as reported in our previous study (Hashimoto et al. 2011). As shown in Table 1, high sequence similarity was found for each of the proteins between this strain and ATCC53264 or 1306-03 (GenBank: AAA21884 – 21887), and probably ATCC53524 or 1306-21, which is a derivative strain of ATCC53264 (Wong et al. 1990).

Table 1. Amino acid sequences of the peptide antigens for the antibodies used in this study (JCM9730), together with the sequence of the corresponding part for ATCC53264. The non-identical residues are indicated with shadowing.

Protein and strain		Amino acid sequence
CesA	JCM9730 (antigen)	S G Q T Q E G K I S R A A S
	ATCC53264	S G Q T Q E G K I S R A A S
CesB	JCM9730 (antigen)	S P D L Y T W R D R P N K
	ATCC53264	S P D L Y T W R D R P Y K
CesC	JCM9730 (antigen)	P S I D G G L G F R S R S G E H
	ATCC53264	P S I D G G L G F R S R S G E H
CesD	JCM9730 (antigen)	T R D I D A E D L N S
	ATCC53264	T R D I D A E D L N S

The centrifuged cells described above were resuspended in a buffer of 10 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.02% NaN_3, 50 μg/mL chloramphenicol. Then, the cell suspension was mixed with the SDS-PAGE sample buffer. After incubating at 4°C for overnight, the sample was analyzed with a precast gel with a gradient of 5-20% acrylamide (SuperSep Ace, Wako Pure Chemicals Industries Ltd., Japan). The band pattern was transferred from the gel to a PVDF membrane (Immobilon-P, Millipore Inc.), and then the membrane was incubated with each of the primary antibodies against CesA, CesB, CesC, and CesD protein. Finally, the protein band was visualized on the PVDF membrane by a chemical luminescence

method with ECL select (GE Healthcare Inc.) and recorded by a CCD camera (EZ-capture, ATTO Inc., Japan).

Preparation of the cells for immunolabeling

The centrifuged cells were resuspended in CBS (citrate buffered saline: 50 mM sodium citrate buffer (pH 5.0), 136 mM NaCl, 2.7 mM KCl) and then incubated in 2% PFA in CBS at 4°C overnight to chemically fix the cells. Then, the gently centrifuged cells (1000×*g* for 10 min at RT) were resuspended in PBS (phosphate buffered saline: 10 mM phosphate buffer (pH7.4), 136 mM NaCl, 2.7 mM KCl) with 0.1 M glycine for quenching the PFA. The cell suspension was dropped on the glass coverslip, which was made to be hydrophilic in advance by dipping in 1 mg/mL poly-L-lysine solution at RT for 30 min. The coverslip carrying the cells was processed with the following procedures of lysozyme treatment and permeabilizing treatment, prior to the antibody treatment.

The cells on the coverslip were treated with 1 mg/mL lysozyme in TE buffer (100 mM Tris-HCl (pH 6.7), 5 mM EDTA) at 37°C for 1 h. After four repeated washes with PBS, the cells were permeabilized with 1% IGEPAL CA-630 (MP Biomedicals LLC; equivalent to a detergent Nonidet P-40) in PBS at 30°C for 30 min. The cells were then washed four times with PBS for 5 min each time. Some of these treatments were skipped to explore the subunit localization in the cell.

Immunolabeling of the cells

Prior to the antibody treatment, the cells on the coverslip were incubated in 1% BSA and 1% Blocking Reagent (Roche Inc.) in PBS at RT for 1 h for blocking. Then, the cells were treated with the primary antibody solution, which was diluted 500-fold in the blocking buffer, at 4°C for overnight with gentle

shaking. After four washes with PBS every 5 min, the cells were treated with 5 µg/mL of the fluorophore-conjugated anti-rabbit IgG (Alexa fluor 488, Thermo Fisher Scientific Inc.) in the blocking buffer at RT for 2 h in the dark. The cells on the coverslip were washed with PBS four times every 5 min. The coverslip was taken out to wash the side without the cells in pure water, and then placed on the slide glass to seal the cells in the anti-fading reagent (SlowFade, Invitrogen Inc.). Control experiments were done for the fixed *Acetobacter* cells by using either no primary antibody or fluorophore-conjugated anti-mouse IgG (Alexa fluor 488, Thermo Fisher Scientific Inc.) for the secondary antibody.

The cells on the slide glass were observed on an IX71 microscope (Olympus Inc., Japan) with an oil immersion lens. Epi-fluorescence mode with a mercury lamp and the filter set FITC-2024B (Semrock Inc., USA) was used for recording the fluorescence image by a CCD camera (DP73, Olympus Inc., Japan). The same region of interest was also recorded with the phase-contrast mode for subsequent merging with the fluorescence image.

SDS-freeze replica labeling

The cells collected from the pellicle as described above were quickly frozen on the gold sample career by dipping into liquid ethane at −175°C with a Leichert KF-80 system (Leica Inc.). The freeze-fracture replica of these cells without chemical fixation was prepared using a BAF-400D system (Balzers Inc.). The fracture was performed at −113°C, and then platinum/carbon was evaporated on the fractured surface at an angle of 45° followed by rotary carbon coating to support the platinum replica. The prepared replica was treated in the lysozyme solution (1 mg/mL lysozyme in 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) for 4 h at RT, and subsequently in the lysis solution (2.5% SDS, 10 mM Tris-HCl (pH

8.0)) for 2 h at RT. After washing three times with PBS, the replica was then incubated in the blocking solution (1% BSA in PBS) for 30 min at RT, and then treated with the primary antibody diluted in the blocking buffer overnight at 4°C. The replica was then washed in PBS with 0.05% Tween-20 (PBST) and treated with the secondary antibody (anti-rabbit IgG conjugated with 15-nm colloidal gold, British BioCell International, UK) for 1.5 h at RT. Finally, the replica was treated with 0.5% glutaraldehyde in PBS for 15 min at RT, and then transferred on the carbon-coated copper grid after washing with water.

The replica on the grid was observed by a JEM-2000EXII (Jeol Inc., Japan) electron microscope and the images were recorded with photo-emulsion (FG film, FujiFilm Inc., Japan), which was developed by Korectol (FujiFilm Inc., Japan) for 4 min at 20°C.

RESULTS

CesA is present in the linear array in the bacterial cells

The results of the western blot analysis with the antibodies used in this study are shown in Figure 1. These antibodies basically showed cross-reactivity to the proteins extracted from *Acetobacter* in the three different strains used in this study (ATCC 53524, ATCC53264, and JCM9730). We then used these antibodies for immunolabeling fluorescence microscopy, as shown in Figure 2.

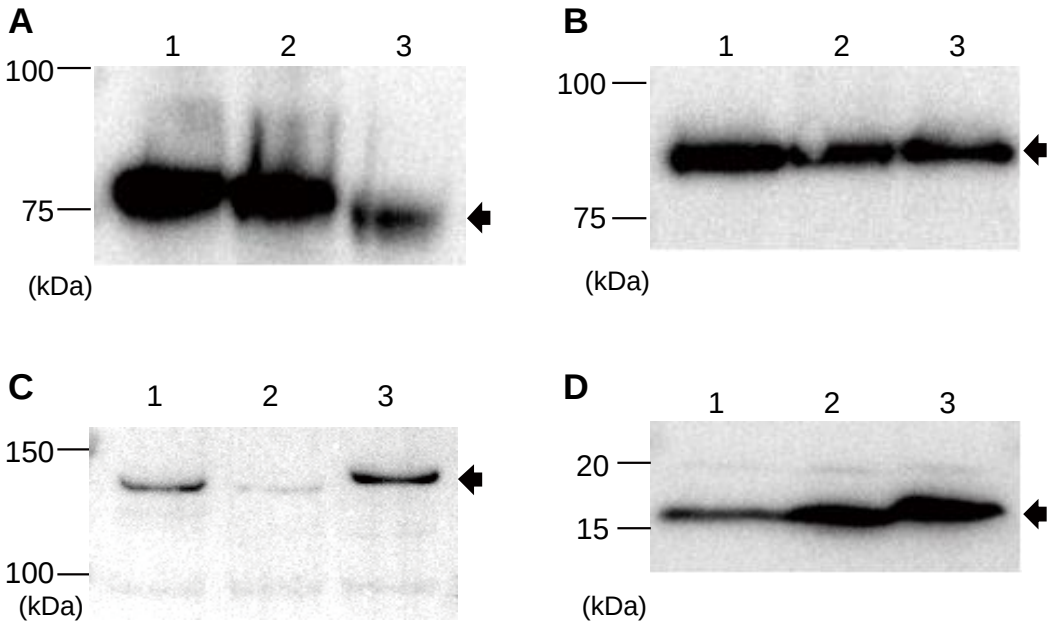

Figure 1.

Western blot analysis with SDS-PAGE for the whole cell sample of *Acetobacter* cells. A, B, C, and D show the results with the antibody against CesA, CesB, CesC, and CesD protein, respectively. The sample of ATCC53524, ATCC53264, and JCM9730 was loaded into the lane 1, 2, and 3, respectively. Roughly the same number of cells, measured by the optical density at 600 nm, were loaded. The arrow indicates the band of interest.

As a result of optimizing the pretreatment of the cells (fixation, lysozyme treatment, and detergent treatment) as well as the antibody treatment, we could successfully label CesA and CesD proteins as a linear array in the cell (Figure 2A) whereas no labeling was found in negative controls (Figure 2B). Such a linear labeling pattern was not found for the immunolabeling of CesB and CesC. The linear labeling pattern of CesA and CesD was observed for all of the strains used in this study. This clearly indicates that CesA and CesD proteins are the subunits included in the linear TC of *Acetobacter*. Furthermore the linear signal was sometimes found at the lateral edge of the cell on the micrograph, indicating that the labeled protein is not on the inside but rather at the boundary of the *Acetobacter* cell. Therefore, the linear immunolabeling pattern shown in Figure

2A provides the experimental evidence that CesA and CesD are included in the linear TC on the cell membrane, the bacterial CSC.

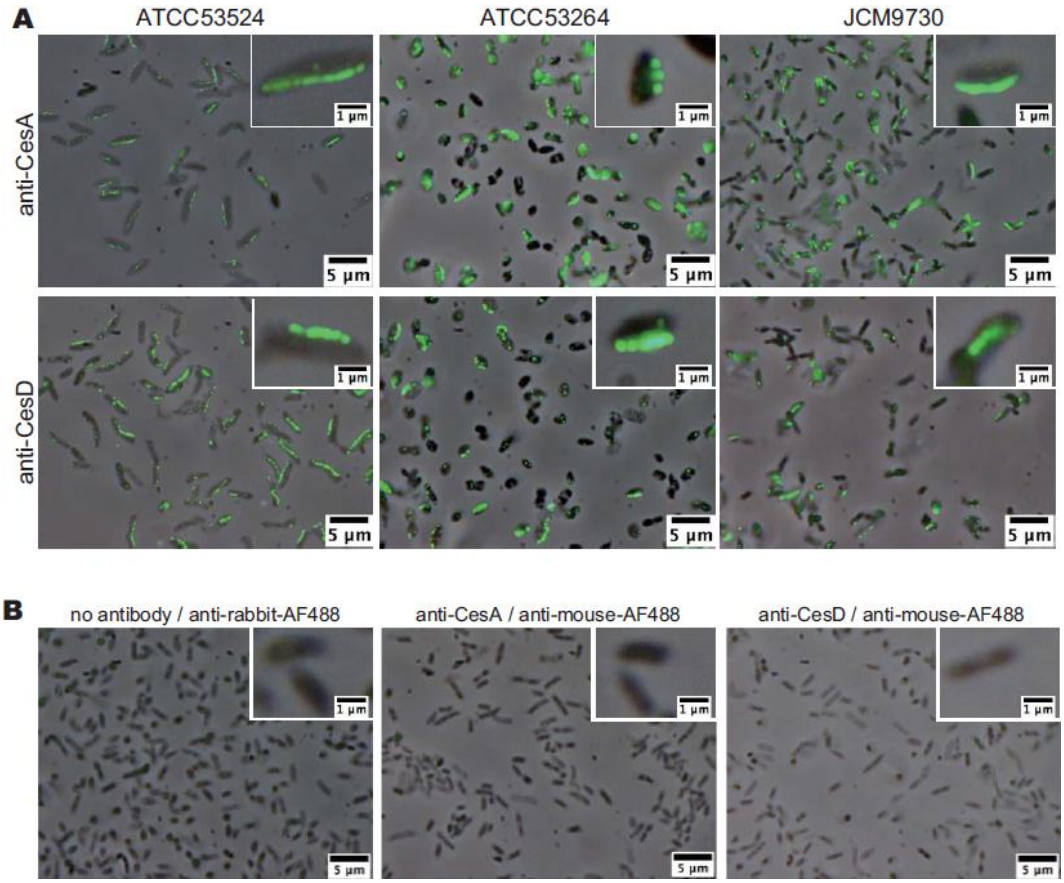

Figure 2.

Fluorescence micrographs of *Acetobacter* cells with immunolabeling by the antibodies against CesA and CesD proteins. The phase-contrast images and the epi-fluorescence image are merged. (A) Three different strains (ATCC53524, ATCC53264, and JCM9730) were labeled using the identical protocol with a correct choice of the antibodies. The inset shows the image at a higher magnification. (B) Control experiments with a strain ATCC53524. Combination of the primary/secondary antibodies was used as indicated. Almost no labeling was found in neither conditions.

Change of the immunolabeling efficiency for CesA and CesD protein.

For efficient immunolabeling, the cells are usually treated with an adequate procedure prior to labeling. In the case of the bacterial cell, lysozyme

262 treatment is commonly used for disintegrating the peptidoglycan layer beneath the
263 outer membrane, and detergent treatment is used for permeabilizing the outer and
264 inner membrane. Therefore, in principle, the protein exposed to the outside of the
265 cell will be labeled without any pretreatment. We then surveyed the change in the
266 immunolabeling efficiency depending on the pretreatment applied to explore the
267 location of CesA and CesD proteins, which were successfully immunolabeled in
268 this study. The strain ATCC53524 was used for this purpose given the fact that
269 this strain showed the highest immunolabeling efficiency.

270 We tested five different pretreatments, in addition to the optimized
271 condition shown above (Figures 3A and 4A): (i) no treatments, (ii) EDTA
272 treatment, (iii) detergent treatment, (iv) EDTA treatment followed by detergent
273 treatment, and (v) lysozyme treatment (Figures 3 and 4, and summarized in Table
274 2). First, the cells with no pretreatment showed almost no immunolabeling for
275 neither CesA nor CesD (Figures 3B and 4B), indicating that CesA and CesD are
276 not exposed to the outside of the cell. Notably, EDTA treatment alone allowed for
277 the immunolabeling of CesD but not CesA (Figures 3C and 4C). Given the
278 relatively mild disturbance of the outer membrane only by depletion of divalent
279 cations with EDTA, CesD is probably located in the periplasmic space and was
280 immunolabeled due to access of the antibody. By contrast, CesA protein is a
281 transmembrane protein, with its carboxyl terminal (the epitope of the antibody
282 used in this study) facing to the cytoplasm. Therefore, it is reasonable that CesA
283 was not immunolabeled for cells whose outer membrane is mildly disturbed by
284 EDTA alone, which is not harsh enough to allow for cell lysis.
285

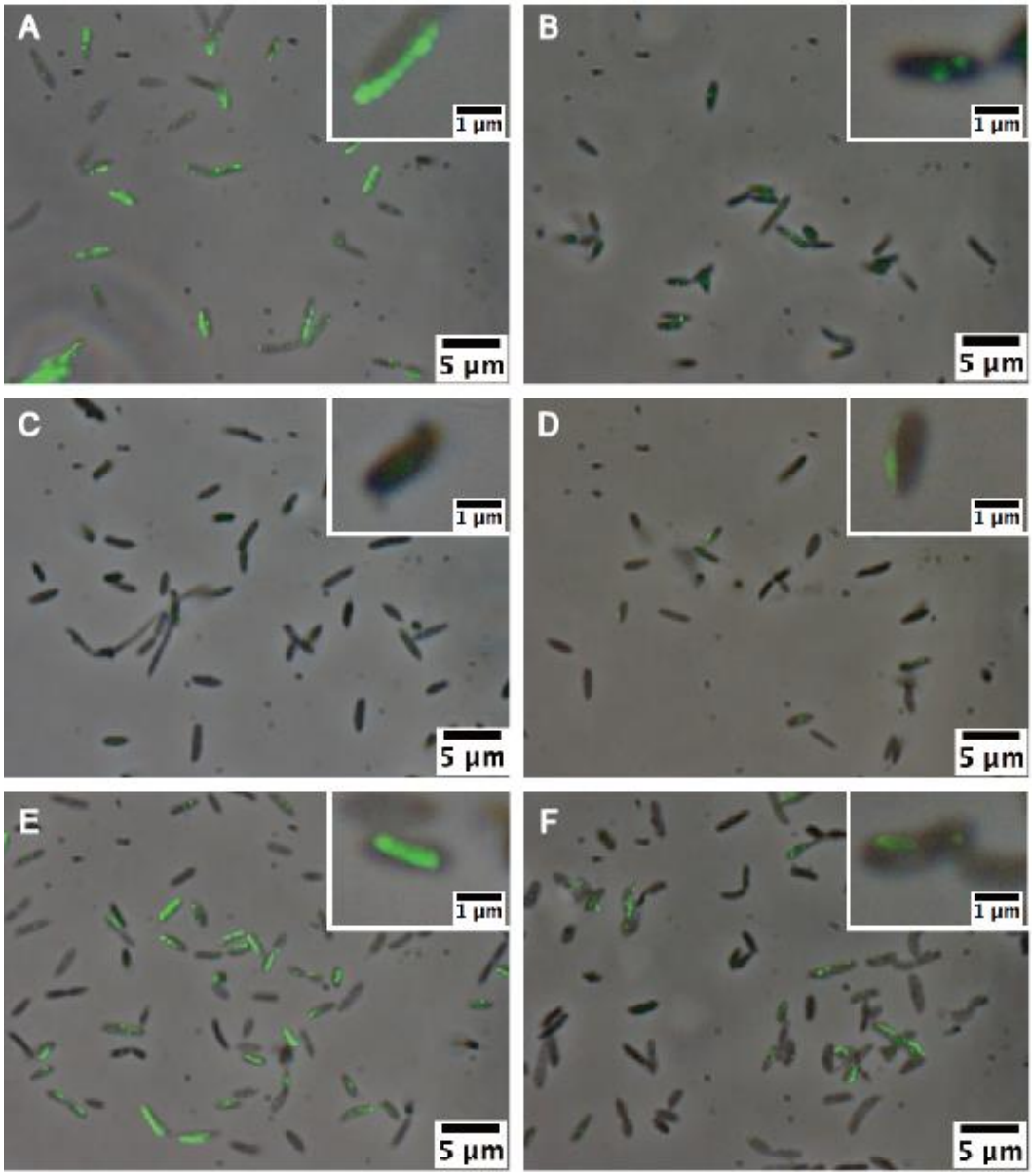

Figure 3.

Fluorescence micrographs with immunolabeling of the strain ATCC53524 by the antibody against CesA protein, merged on the phase-contrast image. Pretreatment of the cell prior to the primary antibody treatment was as follows: (A) lysozyme treatment followed by detergent treatment (the optimized condition in this study), (B) no pretreatment, (C) EDTA treatment, (D) detergent treatment, (E) EDTA treatment followed by detergent treatment, (F) lysozyme treatment. The inset shows the image at a higher magnification.

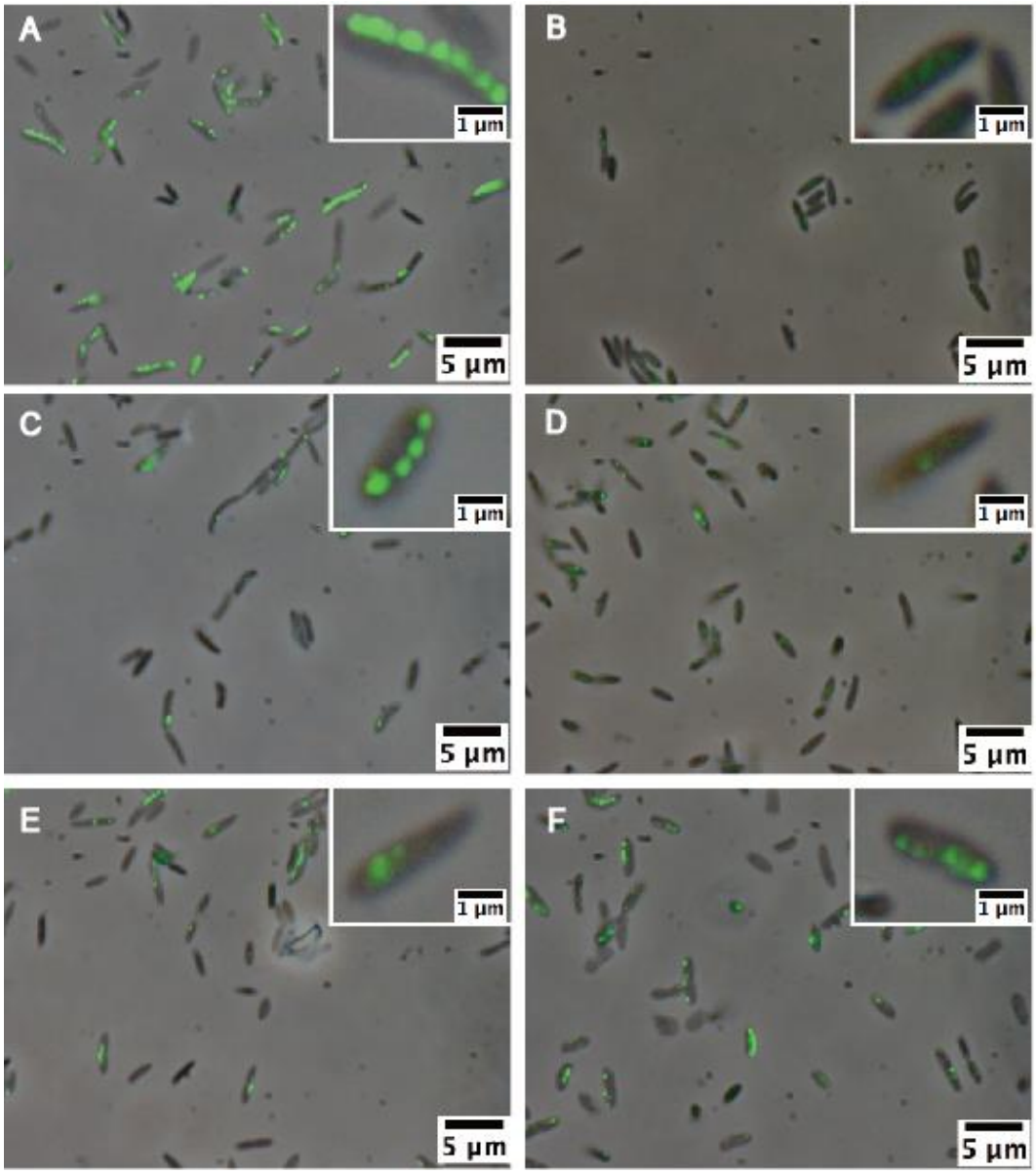

Figure 4.

Fluorescence micrographs with immunolabeling of the strain ATCC53524 by the antibody against CesD protein, merged on the phase-contrast image. Pretreatment of the cell prior to the primary antibody treatment was as follows: (A) lysozyme treatment followed by detergent treatment (the optimized condition in this study), (B) no pretreatment, (C) EDTA treatment, (D) detergent treatment, (E) EDTA treatment followed by detergent treatment, (F) lysozyme treatment. The inset shows the image at a higher magnification.

Table 2. Summary of the immunolabeling microscopy observations

Pre-treatment after PFA fixation	CesA	CesD
Lysozyme treatment + Detergent treatment	+++	+++
No treatment	−	−
TE* treatment	−	+
Detergent treatment	−	−
TE treatment + Detergent treatment	++	++
Lysozyme treatment	++	++

−: Almost no labeling was found

+: A small number of the cells were labeled

++: A substantial number of the cells were labeled

+++: Most of the cells were labeled

*: Tris-EDTA buffer (100 mM Tris-HCl (pH 6.7), 5 mM EDTA), the same buffer used for the lysozyme treatment

Detergent treatment alone did not allow for the immunolabeling of CesA and CesD, in contrast to the expectation (Figures 3D and 4D). However, EDTA treatment prior to detergent treatment dramatically improved the immunolabeling efficiency for both CesA and CesD (Figures 3E and 4E). This indicates that the permeabilization by the detergent is not sufficient for disturbing the outer membrane of *Acetobacter* to introduce the antibody to the inside of the cell (periplasm and cytoplasm).

A substantial number of cells were immunolabeled when treated with lysozyme alone for both CesA and CesD (Figures 3F and 4F). Given that CesD is

localized in the periplasm, as shown above, the lysozyme treatment without permeabilizing the inner membrane was sufficient to immunolabel CesD protein. However the substantial immunolabeling of CesA protein from such pretreatment requires a speculative interpretation given that the carboxyl terminal of CesA protein (the epitope of the antibody used in this study) is on the cytoplasmic side and prevents access of the antibody unless the inner membrane is permeabilized. We consider that this observation reflects weak but nevertheless significant cell lysis due to the lysozyme treatment.

DISCUSSION

This study provides evidence that CesA is included in the TC of bacterial cells, which had already been reported for plant cells (Kimura et al. 1999). Based on fluorescence immuno-microscopy, this study also showed that CesD is included in the linear TC, as reported previously (Sunagawa et al. 2013). These observations are not sufficient to conclude that the CesA and CesD proteins are colocalized in the TC. Direct immunolabeling with a fluorescence dye-labeled primary antibody should provide a clearer conclusion for the colocalization of CesA and CesD proteins in the TC. Neverthelss, the linear labeling pattern observed for CesA and CesD in this study is striking enough to propose that CesA and CesD are colocalized in the TC of *Acetobacter*, regardless of whether their interaction is direct or indirect.

The structural models for the CesA/CesB complex (Morgan et al. 2016, Morgan et al. 2013, Morgan et al. 2014) and CesD (Hu et al. 2010) also support the functional link between CesA and CesD, given that the former generates cellulose from UDP-glucose and the latter includes cellulose chains in the channel

formed by its homo-octamer. It is then proposed that CesD functions downstream of the CesA/CesB complex in the process of cellulose biosynthesis, and that they are spatially close. This hypothesis is consistent with the observation that immunolabeling of CesA and CesD proteins showed a linear pattern in the cells in this study.

We also attempted the immunolabeling of CesB and CesC protein in this study although no successful data were obtained. For CesC protein, which is currently the most enigmatic subunit, the reason for the failure is unclear. A possible reason could be related to access of the antibody to the epitope, which is significantly influenced by the stereo arrangement of this subunit in the cell. However, it was unexpected that the immunolabeling of CesB protein did not show a linear labeling pattern as previously observed by SDS-FRL (Kimura et al. 2001). It is noticeable that the antibody against CesB used in this study allowed for the linear TC to be labeled by SDS-FRL (Figure 5), despite a relatively high non-specific labeling probably owing to that it is a polyclonal antibody. This result indicates that this antibody is able to label CesB protein in the SDS-treated freeze-replica prepared from the cells fixed by flash-freezing with no chemicals. Therefore, a possible interpretation for this unexpected result is that the PFA fixation might kill the epitope activity of the CesB protein, for example by changing the protein itself and/or its surrounding environment, so as to inhibit binding of the antibody in immunofluorescence labeling.

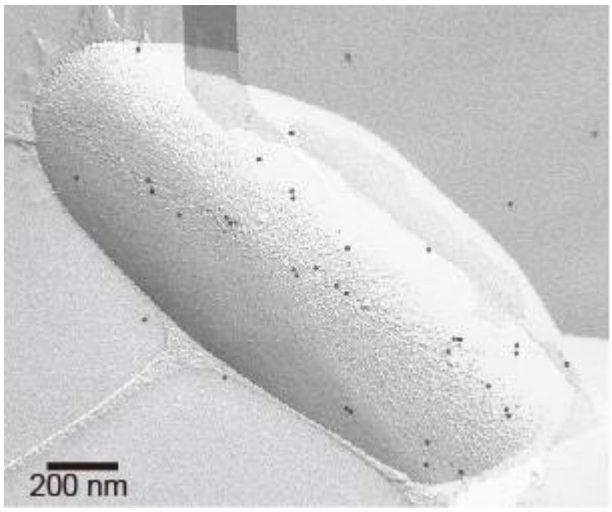

Figure 5.

Electron micrograph of SDS-FRL for the strain ATCC53524 with the antibody against CesB protein. The antibody location was visualized by colloidal gold of a 15 nm diameter. Linear pattern of the labeling was clearly found despite a relatively high non-specific antibody labeling, which is probably due to that the antibody is a polyclonal antibody against peptide.

The antibody against CesA protein, which gave successful immunolabeling as shown in Figure 2A, was also used for SDS-FRL to visualize CesA protein in *Acetobacter* TC. However to date, no labeling for CesA protein was found on the replica despite successful labeling for CesB protein as shown above. This is probably due to that CesA protein in the inner membrane was detached from replica of the outer membrane by solubilization with SDS. Significant improvement will be required for successful SDS-FRL with the antibody against CesA protein.

Compiling the results of this and previous studies, we propose a hypothetical model for the TC of *Acetobacter* as shown in Figure 6. The CesA/CesB complex is embedded in the inner membrane, given that the ligands (UDP-glucose and c-di-GMP) are cytosolic molecules and the product cellulose is extruded outside through the membrane-spanning channel (Morgan et al. 2013).

CesC is depicted as the cellulose-translocating channel in the outer membrane according to the currently accepted model (McNamara et al. 2015, Saxena et al. 1994). No immunolabeling from the lack of pretreatment, and weak immunolabeling from EDTA treatment alone for CesD protein indicated that CesD is located in the periplasmic space. The result of a biochemical study using marker enzyme assays also support this hypothesis (Iyer et al. 2011). Given that the function of CesD is carried out downstream of CesA as discussed above, CesD protein is located close to the exit of the cellulose-translocation channel of CesA protein in the periplasm, as proposed based on a previous structural analysis of the *Acetobacter* CesA/CesB complex with electron microscopy (Du et al. 2016).

Given that one CesA/CesB complex produces one cellulose chain (Morgan et al. 2013), and the CesD oligomer includes four chains in its inner pore (Hu et al. 2010), the model in Figure 6a represents only one CesA/CesB complex, and the other three complexes are not shown for visual clarity. A combination of these molecules could be the functional unit to produce the primary assembly of the polymerized cellulose chains prior to microfibril formation, which has been proposed as a “mini-sheet” in a previous study (Cousins and Brown Jr. 1995). The linear array of this whole complex should be visualized as the linear type TC in *Acetobacter* (Figure 6b).

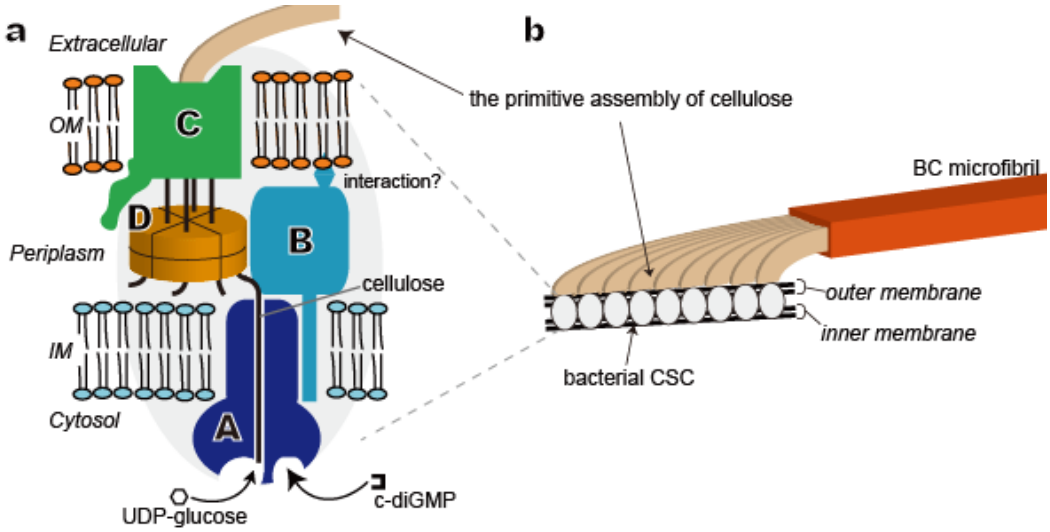

Figure 6.

A schematic model for the cellulose synthase complex of *Acetobacter*. In the schematic diagram of the subunit location in one complex (a), CesA and CesB are depicted as monomers while CesD is illustrated as an octamer through which four cellulose chains pass, as reported previously (Hu et al. 2010). CesC is located in the outer membrane according to the currently accepted model (McNamara et al. 2015, Saxena et al. 1994). The terminal complexes are probably formed by the linear array of these complexes as shown in (b). OM: outer membrane; IM: inner membrane

The SDS-FRL experiment also provided insight about CesB protein. Given the smoothness of the fractured surface, the linear TC of *Acetobacter* is found in the P-face (the extracellular surface of the inner leaflet of the lipid bilayer) of the outer membrane (Kimura et al. 2001). The successful immunolabeling of CesB protein by SDS-FRL indicates that this protein remains with the replica even after SDS-treatment, indicating that CesB protein significantly interacts with the outer membrane from the periplasmic side. This interaction is likely important for guiding the cellulose chain to the extracellular side and/or the crystallization of cellulose chains into a microfibril. Further SDS-FRL experiment with other antibodies will shed light on the locations of the other subunits.

CONCLUDING REMARKS

This study demonstrated that CesA, the core catalytic subunit of cellulose synthase, is the molecule included in the linear-type TC or the CSC of *Acetobacter*. Structural analysis of these proteins has recently started providing many insights about the enzymatic mechanism of cellulose synthase as well as other well-known membrane proteins such as ion/water channels and transporters. However, for cellulose synthase, which functions in the assembly of polymer chains into a supermolecular aggregation, the structural analysis of the protein complex at a cellular/subcellular scale is important for understanding the underlying mechanism. Further studies with microscopy will play an important role for shedding light on the mechanism of cellulose chains assembly into the microfibril.

Acknowledgements

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